Tumor Associated Macrophages Mitigate Oncolytic Herpes Simplex Virus Anti-Tumor Efficacy in Ewings Sarcoma

Nicholas Denton[1], Chun-Yu Chen[1], Brian Hutzen[1], Jennifer Leddon[1], Pin-Yi Wang[1], Thomas Scott[1], Mark Currier[1], Tim Eubank[3], Timothy P. Cripe[1,2]

[1]Center for Childhood Cancer and Blood Diseases and [2]Division of Hematology/Oncology/Blood and Marrow Transplant, Nationwide Children's Hospital, Columbus, OH 43205. [3]Department Pulmonary, Allergy, Critical Care, & Sleep Medicine, The Ohio State University, Columbus, OH 43210.

Introduction

Cancer is the cause of 1 in 4 deaths in the United States and is the leading cause of disease-related death in children. Ewing sarcoma (EWS) is a highly aggressive pediatric and adolescent bone tumor that recurs or metastasizes in 50% of pateints.[1-2] Recurrent and metastatic EWS tumors are often lethal because these tumors are highly resistant to the current therapy regimen of surgical resection, irradiation, and chemotherapy. Therefore, there is an unmet demand for novel therapies that can destroy these aggressive pediatric and adolescent tumors.

Oncolytic viruses (OVs), such as the rRp450 herpes simplex virus, are promising anticancer therapeutics designed to selectively replicate in cancer cells.[3-6,11-12] The rRp450 virus is attenuated to selectively replicate in tumor cells through the depletion of the UL39 virus gene encoding the large subunit of infected cell protein 6 (ICP6) of viral ribonucleotide reductase (Fig. 1A). This disruption of ribonucleotide reductase prevents the rRp450 from producing the

dNTP DNA subunits required for virus replication in healthy cells. However, highly proliferative cancer cells produce their own stock of dNTP DNA subunits during DNA replication that rRp450 is able to utilize in virus replication. The rRp450 virus also has the insertion of rat gene CYP2B1 encoding rat cytochrome P450 enzyme to cleave cyclophosphamide into its active form, but this feature was not tested farther in this study.

While OV anti-tumor efficacy is partially caused by direct infection and lysis of cancer cells, stimulation of an anti-cancer immune response also contributes to virus-mediated efficacy.[7] Previously we have characterized the response of two highly aggressive human EWS xenograft tumors to rRp450 oncolytic herpes simplex virus (oHSV) and found that the A673 cell line is vulnerable to rRp450 *in vitro* and virus replication increases over time in both the *in vitro* A673 cell lines and *in vivo* A673 xenograft tumors (Fig. 1B). The 5838 cell line, however, is highly resistant to rRp450 oncolysis *in vitro* and virus replication diminishes over time in both the *in vitro* 5838 cell lines and *in vivo* 5838 xenograft tumors. However, we observe a disconnect between the *in vitro* rRp450 cytotoxicity and virus replication to the *in vivo* anti-tumor response; the majority of 5838 tumors respond completely after rRp450 infection while A673 tumors only experience a slight inhibition in tumor progression after virus infection. These disconnect between *in vitro* and *in vivo* response to oncolytiv virus therapy suggests that oncolytic virus anti-tumor efficacy in these EWS xenograft tumors is highly dependent on the influence of the tumor microenvironment.

Immunologic responses to infections are known to be modulated by macrophages through various cytokines and chemokines and it is now appreciated that the majority of tumors are replete with tumor associated macrophages (TAMs).[8-10] Macrophages are generally activated between two polarized activation states: classically activated M1 and alternatively activated M2

macrophages. M1 classically activated macrophages are considered to be anti-tumor macrophages due to their secretion of cytotoxic reactive oxygen species and pro-inflammatory cytokines. M2 alternatively activated macrophages are considered to be pro-tumor macrophages due to their expression of growth factors and pro-tumor immunosuppressive cytokines, such as IL-10 and TGF-β.[13-15]

It has been well established that both adult and pediatric tumors are considerably more aggressive when there is higher infiltration of M2 tumor macrophages. There are a considerable number of studies demonstrating that immunosuppressive M2 TAMs inhibit the anti-tumor efficacy of a variety of immunotherapies.[24-26] However, there are no investigations looking into the effect of TAMs on pediatric tumor response to immunotherapy despite the similarities between adult and pediatric tumor macrophages.[21-23] Considering how the tumor macrophages are the predominant myeloid cell population in these Ewing sarcoma models, we hypothesize that TAMs reduce therapeutic efficacy by producing an immunosuppressive tumor microenvironment via IL-10 and TGF-β signaling.

Methodology

Human Ewing sarcoma cell lines were cultured in RPMI media supplemented in 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich). Tumorigenesis was determined with treatment of athymic nude (nu/nu) with 400 ug macrophage depleting clodrosome (liposomal clodronate, Encapsula) or control liposome intravenously (Day -3) and intraperitoneally (Days -2, -1) prior to right flank subcutaneous implantation of EWS cell lines (3.5e6 cells in 100ul 33% matrigel per mouse). Tumor response to oncolytic herpes simplex virus was determined with human Ewing sarcoma xenografts implanted into athymic nude mice and macrophages were depleted using liposomal clodronate prior to intratumoral injection of

rRp450 oncolytic HSV (e7 pfu in 100 ul PBS) or control PBS (Days 0, 2). Tumors were allowed to grow (50-150 mm^3) prior to treatment for tumor progression and survival measurement; tumor volume was calculated ($V=(L)x(W^{2)}x(pi/6)$) with survival endpoint at tumor volume >2000 mm^3. *In vitro* and *in vivo* virus replication was determined through plaque assay of 100 ul serially diluted, rRp450 infected EWS cell and tumor samples applied to a fully confluent 12 well plate of vero cells, incubating in 37^0C for 3 days in overlay media, and staining with crystal violet prior to plaque counting. $F4/80^+$ tumor macrophages were positively selected from tumor samples through 33%-66% percolli gradient followed by $F4/80^+$ magnetic bead separation; sample RNA was harvested and reverse transcribed for qPCR analysis of tumor inflammatory signaling and M1/M2 macrophage gene profiles. TGF-β cytokine superfamily receptor signaling was inhibited with 150 ug A83-01 (Sigma-Aldrich) small molecule treatment given intraperitoneally thrice weekly prior to intratumoral injection of rRp450.

Results

Tumor associated macrophages are the predominant leukocyte population in a majority of aggressive pediatric sarcomas. To determine if macrophages play a role in tumor progression and tumorigenesis in Ewing sarcoma xenograft models we depleted the macrophages in athymic nude mice using liposomal clodronate (clodrosome, Encapsula) prior to subcutaneous EWS cell line implantation. Macrophage depletion significantly inhibited tumorigenesis (Fig. 2A) after implantation of A673 into athymic nude mice. These results suggest that the oncolytic HSV-resistant A673 xenograft tumorigenesis is partially dependent on macrophages. Tumorigenesis in the oHSV-sensitive 5838 xenograft was not altered by macrophage depletion (Fig. 2B).

To determine if macrophages play a role in oncolytic herpes simplex virus resistance in EWS xenograft models we allowed the xenograft tumors to grow to a treatable tumor size prior to macrophage depletion with clodrosome, followed by rRp450 treatment. When A673 xenograft tumors were permitted to grow to 50-150 mm^3 in volume clodrosome treatment did not significantly inhibit tumor progression, but tumor response to rRp450 oncolytic herpes simplex virus improved dramatically (Fig. 3A). No change in virus titer was observed in the macrophage depleted tumors, suggesting that the improved anti-tumor efficacy of rRp450 in clodrosome treated A673 xenografts is not due to improved direct oncolysis of the tumor cells but due to other changes in the tumor microenvironment. Clodrosome treatment did not inhibit anti-tumor efficacy of rRp450 for the oHSV-sensitive 5838 Ewings sarcoma xenograft tumors (Fig. 3B).

Macrophages are generally activated either towards a M1 anti-tumor macrophage or a M2 pro-tumor macrophage polarization state; to determine if the differential anti-tumor response of rRp450 treatment between A673 and 5838 Ewing sarcoma tumors following macrophage depletion was due to a difference in the tumor macrophage activation state we extracted the tumor macrophages from our EWS xenograft models and compared the gene expression profiles of macrophages from our oHSV-sensitive 5838 and oHSV-resistant A673 xenografts. The tumor macrophages from the oncolytic virus-resistant A673 xenografts displayed a higher expression of pro-tumor M2 macrophage associated genes compared to the macrophages from the OV-sensitive 5838 xenograft tumors following rRp450 infection (Fig. 4). Likewise, the macrophages from the OV-sensitive 5838 tumors had higher expression of anti-tumor M1 macrophage associated genes compared to the macrophages from the OV-resistant A673 tumors following rRp450 infection. Tumor macrophages from A673 xenografts have also demonstrated higher expression of immunosuppressive IL-10 and TGF-β cytokines than 5838 tumor associated

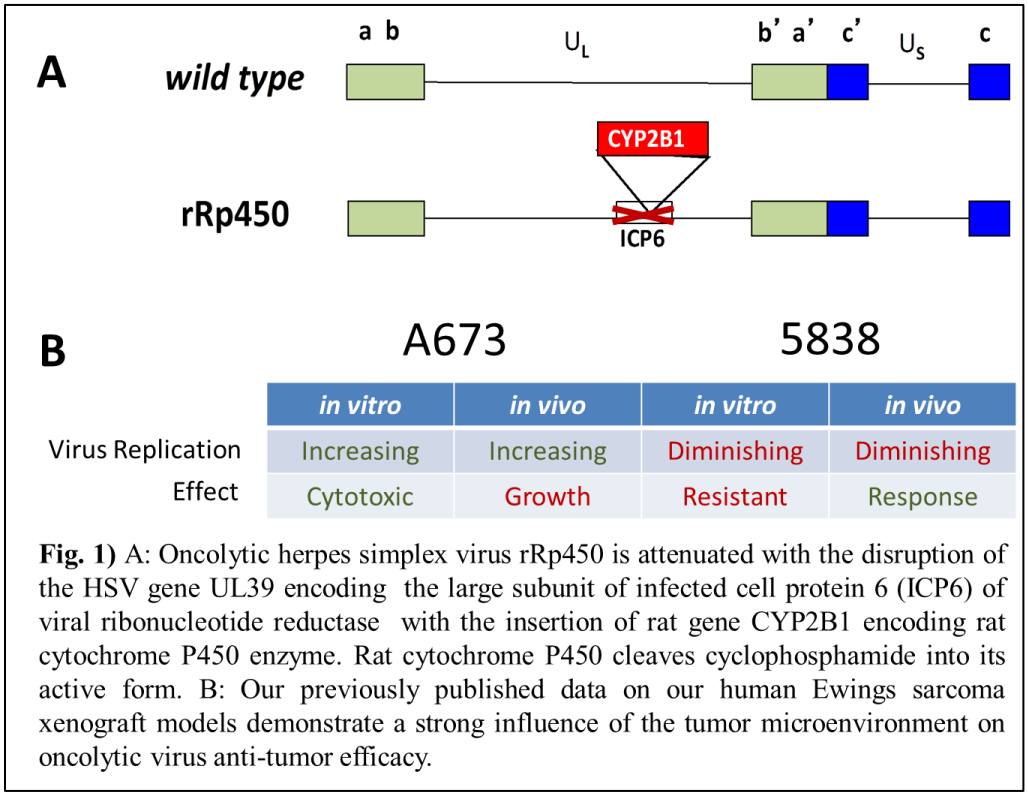
macrophages. Therefore, enhanced OV anti-tumor efficacy in macrophage depleted A673 tumors is predicted to be caused by a decrease of anti-inflammatory signals in the tumor microenvironment such as IL-10 and TGF-β.[18]

TGF-β is known to stimulate macrophages to a pro-tumor M2 activation state and dampen the anti-tumor immune response; to determine the role of TGF-β in Ewings sarcoma resistance to oncolytic virus therapy we inhibited TGF-β signaling with A-83-01 small inhibitor molecule for TGF-β cytokine super family receptor prior to rRp450 treatment. A-83-01 (Sigma-Aldrich) inhibition of TGF-β signaling in OV-resistant A673 xenograft tumors resulted in a modest inhibition of tumor progression and improved response to rRp450 treatment (Fig. 5). It is unclear if TGF-β signal inhibition alone is sufficient to sensitize OV-resistant Ewing sarcoma tumors to oncolytic virus therapy. It is more likely that depletion of M2 tumor macrophages decreases the signaling of a variety of growth factors and immunosuppressive cytokines that contribute to Ewings sarcoma resistance to oncolytic herpes simplex virus therapy.

Conclusions

Macrophages play a significant role in mitigating OV anti-tumor efficacy. Specifically, Ewings sarcoma tumors that promote M2 macrophage polarization are significantly more resistant to oncolytic virus therapy, in part due to TGF-β signaling. However, it is likely that the combination of a variety of growth factors and immunosuppressive molecules secreted by M2 macrophages are involved in Ewings sarcoma resistant to oncolytic virus therapy (Fig. 6). There is also evidence that pro-angiogenic signaling of M2 macrophages hinder the spread of oncolytic viruses throughout the tumor microenvironment.[16-17] Therefore, macrophage depletion may also improve the spread of oncolytic virus in the A673 tumor model. By depleting the macrophages with clodrosome, the A673 tumor-bearing mice are deprived of M2 macrophage secreted growth

factors and immunosuppressive cytokines that normally dampen the anti-tumor immune response induced by oncolytic virus infection. These data suggest that the combination of oncolytic virus therapy with a macrophage modulatory therapy will improve OV anti-tumor efficacy in patients with highly immune suppressive Ewings sarcoma tumors more effectively than combining oncolytic viruses with a small molecule inhibitor for a single redundant immunosuppressive molecule. Farther study is necessary to determine the exact tumor microenvironment changes induced by M2 TAMs that contribute to tumor resistance against OV therapy. There is also the chance to identify a potential M2 macrophage activity biomarker to identify Ewings sarcoma patients that would benefit from a combination therapy of oncolytic herpes simplex virus and macrophage modulation therapy.



	in vitro	in vivo	in vitro	in vivo
Virus Replication	Increasing	Increasing	Diminishing	Diminishing
Effect	Cytotoxic	Growth	Resistant	Response

Fig. 1) A: Oncolytic herpes simplex virus rRp450 is attenuated with the disruption of the HSV gene UL39 encoding the large subunit of infected cell protein 6 (ICP6) of viral ribonucleotide reductase with the insertion of rat gene CYP2B1 encoding rat cytochrome P450 enzyme. Rat cytochrome P450 cleaves cyclophosphamide into its active form. B: Our previously published data on our human Ewings sarcoma xenograft models demonstrate a strong influence of the tumor microenvironment on oncolytic virus anti-tumor efficacy.

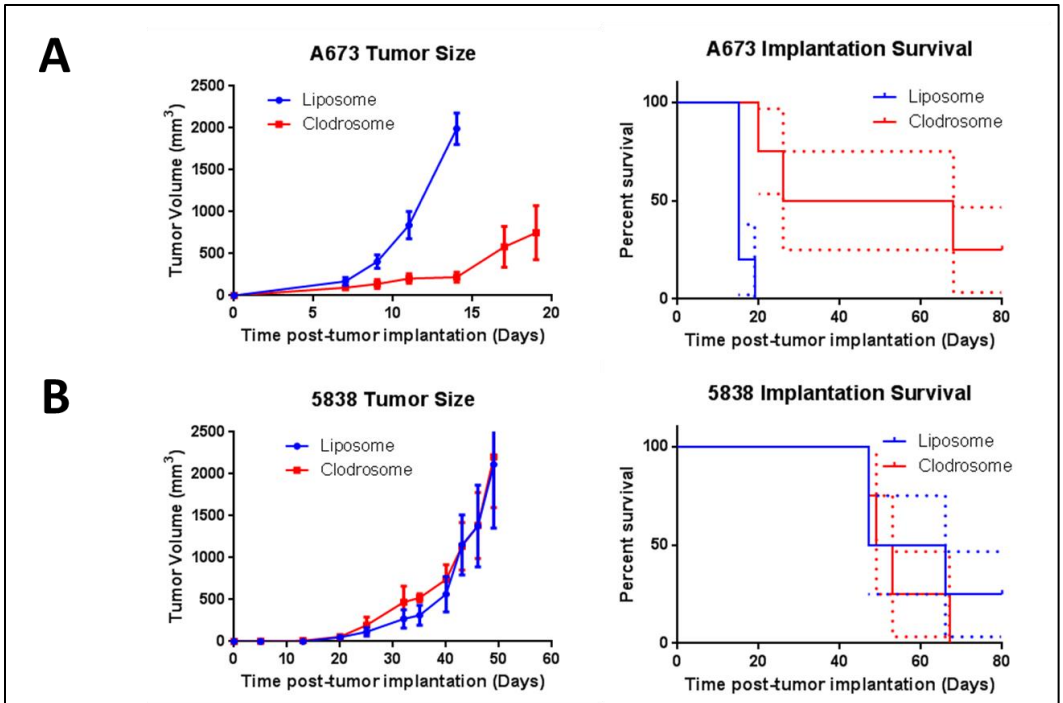


Fig. 2) A: Pretreatment of athymic nude (nu/nu) with 400 ug macrophage depleting clodrosome or control liposome prior to subcutaneous implantation of EWS cell lines. Oncolytic HSV-resistant A673 xenografts are partially dependent on macrophages for tumorigenesis and tumor progression. B: Oncolytic HSV-sensitive 5838 xenograft progression is not affected by macrophage depletion.

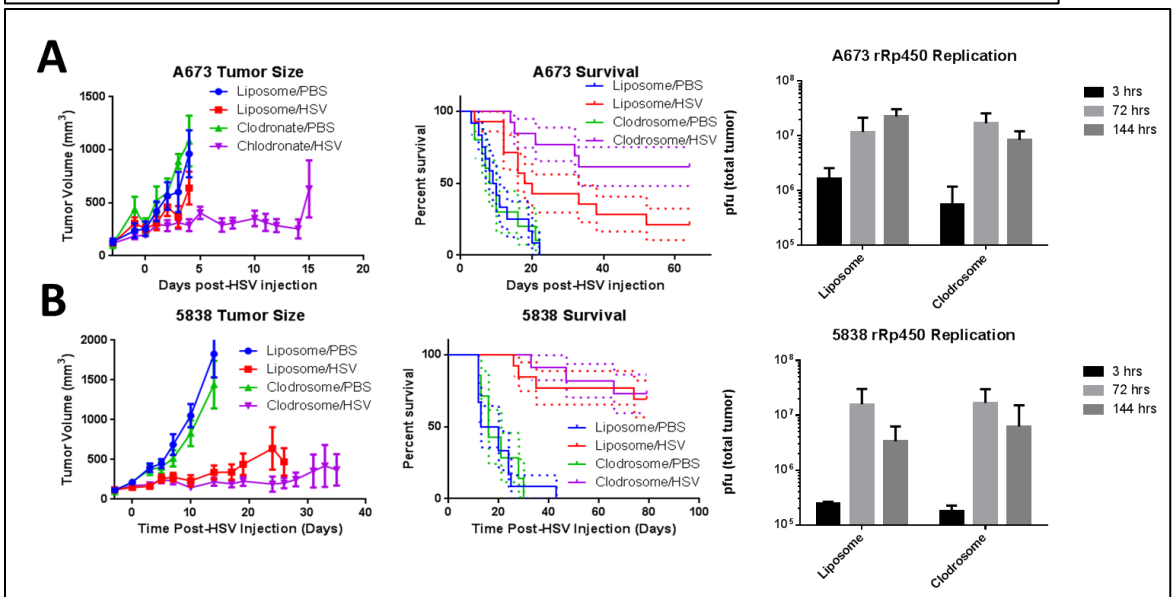


Fig. 3) A: Established EWS xenografts treated with clodrosome or control liposome intravenous (Day -3) and intraperitoneal (Day -2, -1) prior to intratumoral injection of e7 pfu rRp450 (Day 0, 2). A673 xenograft response to rRp450 significantly improves with macrophage depletion. This improved anti-tumor efficacy is not due to changes in virus replication. B: Macrophage depletion does not affect 5838 xenograft response to rRp450.

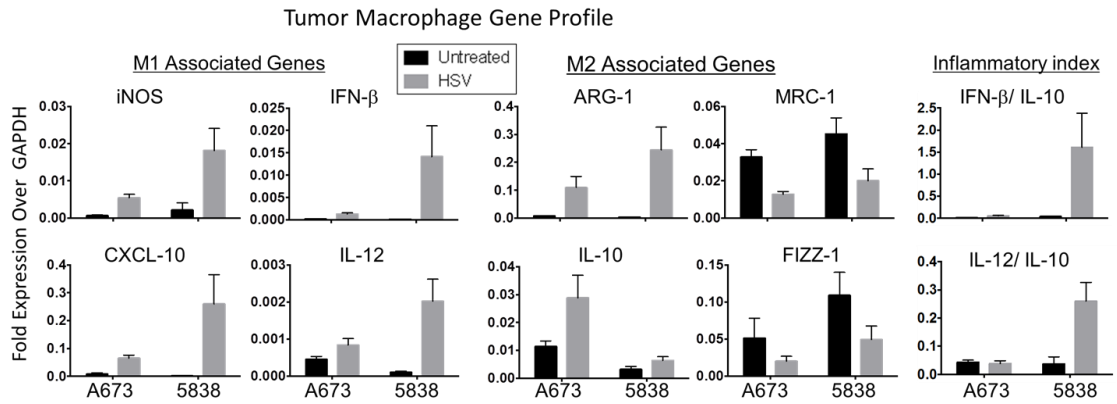


Fig. 4) F4/80⁺ EWS tumor macrophages were positively selected through magnetic bead separation and polarization gene profile was determined through qPCR. Tumor macrophages from virus infected 5838 xenografts have a higher anti-tumor M1 macrophage activation profile and higher inflammatory index than A673 tumor macrophages. Tumor macrophages from virus infected A673 tumor have a higher pro-tumor M2 macrophage activation profile than 5838 tumor macrophages.

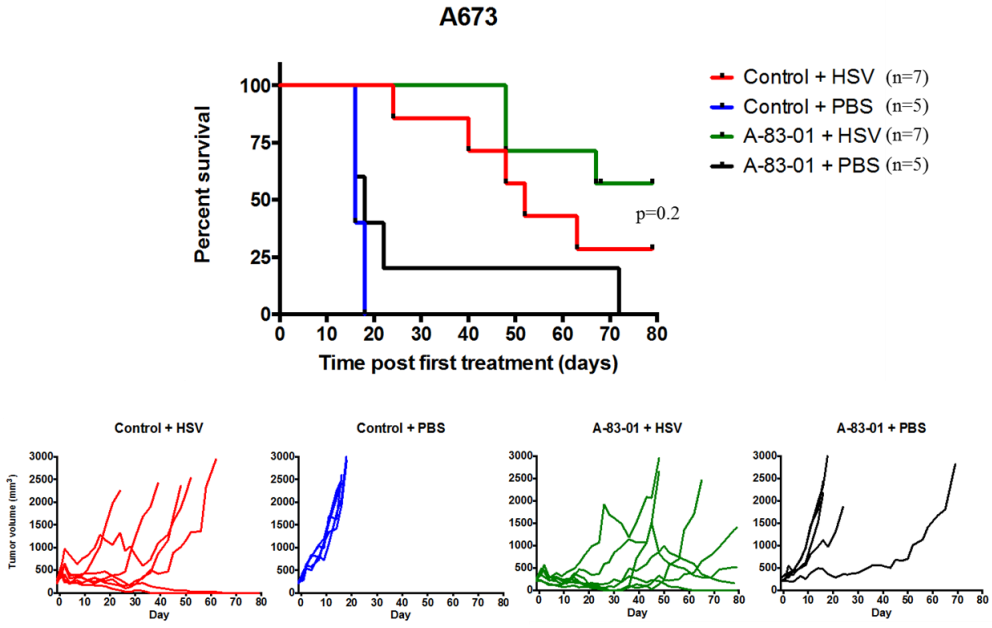


Fig. 5) A673 xenografts were treated with A-83-01 small inhibitor molecule for TGF-β cytokine super family receptor prior to rRp450 treatment. Inhibition of TGF-β signaling partially inhibited A673 xenograft response to rRp450 treatment.

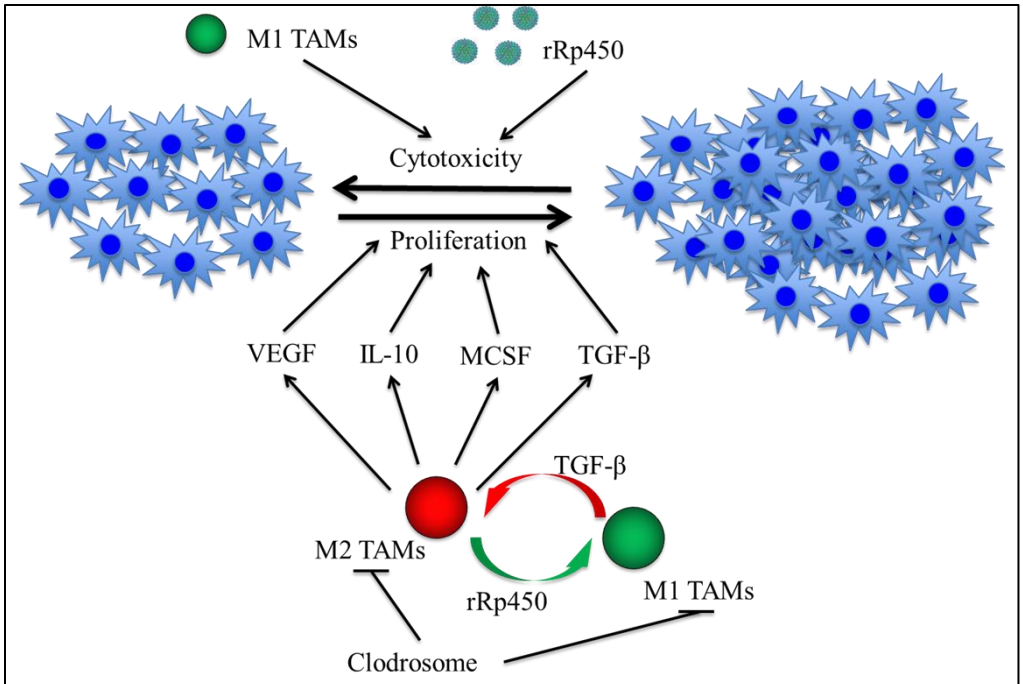


Fig. 6) EWS tumor response to rRp450 is partially dependent on the activation of tumor macrophages. Virus-resistant EWS xenograft tumors have more M2 macrophages, which secrete tumor growth factors and immunosuppressive cytokines that promote tumor proliferation. Therefore, we hypothesize that a combination therapy of oncolytic virus with a M2 macrophage depletion or M1 activation therapy will improve EWS anti-tumor response.

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